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Liposomes assembled from dimeric retinoic acid phospholipid with improved pharmacokinetic properties

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ABSTRACT

All-*trans*-retinoic acid (ATRA) exhibits potent cytotoxicities against different cancer cells by binding to retinoic acid receptors (RARs), which is regarded as the first example of targeted therapy in acute promyelocytic leukemia (APL). However, its extensive clinical applications have been limited because of poor aqueous solubility, short half-life time and side effects. In this report, dimeric ATRA phosphorylcholine prodrug (Di-ATRA-PC) was designed and assembled into nanoliposomes to improve its pharmacokinetic properties. Di-ATRA-PC prodrug was synthesized by a facile esterification and characterized by mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). The Di-ATRA-PC assembled liposomes were prepared by thin film hydration method with ATRA loading efficiency up to 73 wt%. The liposomes have a uniform particle size (73.1 ± 3.6 nm) with negatively charged surface (− 20.5 ± 2.5 mV) and typical lipid bilayer structure as measured by dynamic light scattering (DLS), transmission electron microscope (TEM) and cryogenic transmission electron microscope (cryo-TEM). *In vitro* drug release study confirmed that Di-ATRA-PC liposomes could sustainedly release free ATRA in a weakly acidic condition. Furthermore, cellular uptake, MTT and cell apoptosis analysis demonstrated that the liposomes could be successfully internalized into tumor cells to induce apoptosis of MCF-7 and HL-60 cells. More importantly, *in vivo* pharmacokinetic assay indicated that Di-ATRA-PC liposomes had much longer retention time in comparison with ATRA. In conclusion, Di-ATRA-PC liposomal formulation could be a potential drug delivery system of ATRA with enhanced pharmacokinetic properties.

1. Introduction

All-*trans*-retinoic acid (ATRA), a derivative of vitamin A, plays a critical role in epithelial and hematological cells growth and differentiation *via* binding to specific retinoic acid receptors (RARs) and retinoid X receptors (RXRs) subfamilies (Narvekar et al., 2014; Siddikuzzaman et al., 2011; Masetti et al., 2012). It has been found to inhibit the markers of cell proliferation, such as cyclin D1, epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) (Parthasarathy et al., 1999; Park et al., 2009; Siddikuzzaman and Grace, 2013; Chen et al., 2014). Recently, Wei et al. (2015) reported that ATRA targeted a unique isomerase PIN1, the common key regulator of oncogenic signaling pathways in multiple tumor types. Therefore, ATRA is considered as the first example of targeted therapy in cancer, which simultaneously blocks multiple PIN1-regulated cancer-driving pathways. Till now, ATRA is included in many antitumor therapeutic schemes for the treatment of acute promyelocytic leukemia (APL), Kaposi's sarcoma, head and neck squamous cell carcinoma, breast cancer, ovarian carcinoma, bladder cancer and neuroblastoma (Zuccari et al., 2005; Sun et al., 2005; Orlandi et al., 2003). However, ATRA exhibits drawbacks, such as, poor aqueous solubility, short *in vivo* half-life time, severe side effects and drug resistance (Siddikuzzaman and Grace, 2014; Yao et al., 2013).

To overcome these clinical limitations of ATRA, several strategies of nano-vehicles were developed in recent decades, including polymeric nanoparticles (Tiwari et al., 2011; Park et al., 2008), micelles (Kawakami et al., 2005), emulsion (Hwang et al., 2004) and liposomes (Parthasarathy et al., 1994). These nano-vehicles often possess high therapeutic efficacy and less systemic toxicity. As an example, Tiwari et al. (2011) prepared ATRA loaded poly(ethyleneglycol) (PEG)-poly(L-lactide) and PEG-poly(ε-caprolactone) block copolymer nanoparticles, which had moderate colloidal stability and exhibited around 30% encapsulation efficiency. These nanoparticles could sustainedly release ATRA over a period of two weeks and efficiently induced cellular differentiation in HL-60 cells. Hwang et al. (2004) reported an ATRA-loaded phospholipid microemulsion which had an enhanced solubility, about 25,000-fold higher than that of free ATRA. The kind of formulation demonstrated considerable pharmacokinetic profile and anti-

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cancer effect due to improved solubility and chemical stability. Polymeric micelles incorporated with dipalmitoyl phosphatidylethanolamine (PEG_{750}-DPPE) were an alternative carrier for ATRA delivery. As reported in the literature, PEG_{750}-DPPE based ATRA nanoformulation had high drug loading capacity of 87 wt% and extended storage stability up to 28 days (Wichit et al., 2012).

Liposomes have been considered as one of the most versatile and flexible drug carriers. Many liposomal formulations of drugs have reached the stage of clinical use or trials for the treatment of cancers or other diseases (Rowinsky and Donehower, 1995; Kato et al., 2010). Hydrophobic drugs are usually entrapped in the lipid bilayer of liposomes (Gregoriadis, 1976; Andresen et al., 2005), isolating the drugs from the surrounding aqueous environment and protecting them from structural transformation or chemical degradation. Apart from that, liposomes possess passive targeting ability *via* the enhanced permeability and retention (EPR) effect (Won et al., 2012). Ozpolat et al. (2003) prepared liposomal ATRA formulation which had improved pharmacokinetic properties in comparison with oral ATRA. Goldberg et al. (2002) developed ATRA-encapsulated liposomes with prolonged ATRA serum levels, better bioavailability, acceptable toxicity profile and preliminary anticancer efficacy, as reported in phase I trial stage. Li et al. (2010) prepared folate conjugated liposomes of ATRA, which had the ability of targeting human clonogenic acute myelogenous leukemia cells. However, liposomal formulations have inherent shortcomings including leakage of encapsulated drugs in plasma environment and rapid clearance from blood circulation, which seriously affect the potency of liposomes (Comiskey and Heath, 1990).

In this report, liposomes based on dimeric ATRA phosphorylcholine conjugate (Di-ATRA-PC) were developed in order to improve pharmacokinetic properties of ATRA. The Di-ATRA-PC amphiphilic prodrug was synthesized by conjugating ATRA with sn-1 and sn-2 positions of L-α-glycerophosphorylcholine (GPC) *via* esterification. The prodrug was assembled into liposomes by thin film hydration method. The nanostructure of Di-ATRA-PC liposomes was checked by dynamic light scattering (DLS), transmission electron microscope (TEM) and cryogenic transmission electron microscope (cryo-TEM). The stability and release behavior of the liposomes were investigated by HPLC and DLS. Their intracellular uptake, cytotoxicity and cell apoptosis were further assessed against HL-60 and MCF-7 cells. Furthermore, the pharmacokinetic properties of the Di-ATRA-PC liposomes were evaluated using BALB/c mouse as a model.

2. Materials and Methods

2.1. Materials

All-*trans*-retinoic acid (ATRA, purity > 98%) was obtained from Leinas Chemical Co., Ltd. (Changzhou, China). L-α-Glycerophosphorylcholine (GPC, purity > 99%) was purchased from J&K Scientific Ltd. (Shanghai, China). *N*,*N*′-Carbonyldiimidazole (CDI) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) both were provided by Jingchun Reagent Co., Ltd. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), Iscove's modified Dulbecco's medium (IMDM), phosphate buffered saline (PBS, pH 7.4) and trypsin-EDTA were provided by HyClone (Logan, UT). Fetal bovine serum (FBS) was supplied by Lonsa Co. Ltd. (Richmond, VA). Methyl thiazolyl tetrazolium (MTT) and annexin V-FITC apoptosis detection kit were purchased from Beyotime Co., Ltd. (Shanghai, China). All other chemical reagents used were analytical or HPLC grade, purchased from Nanjing Wanqing Co., Ltd. (Nanjing, China).

Human breast cancer cell line MCF-7 and human acute promyelocytic leukemia cell line HL-60 were received from Typical Culture Preservation Commission Cell Bank, Chinese Academy of Sciences (Shanghai, China). Female BALB/c mice were obtained from Yangzhou University (Yangzhou, China) and used at 7–8 weeks of age. All of the animal related experimental procedures in this manuscript were approved by the Animal Ethics Committee of Southeast University, China.

2.2. Synthesis of Di-ATRA-PC Conjugate

Briefly, to a solution of ATRA (0.601 g, 2.0 mmol) in dichloromethane (5.00 mL), CDI (0.389 g, 2.4 mmol) was added and stirred at 35 °C for 4 h under nitrogen environment. After that, DBU (0.304 g, 2.0 mmol) was added to a solution of GPC (0.206 g, 0.8 mmol) in dimethylsulfoxide (DMSO) (5.00 mL) and stirred at room temperature for 4 h. Then, the two solutions were mixed and stirred at 40 °C for additional 18 h. After reaction completion, the mixture was cooled to room temperature, acidified with 0.1 M acetic acid (12.0 mL) and precipitated into 250 mL diethyl ether. The resulting crude product was purified by column chromatograph eluting with dichloromethane/methanol/water (65/25/4) to obtain the yellow powder (purity 98.0%, yield 23.7%). TOF-MS *m/z*: $[M + H]^+$ calculated 822.50, found 822.49; $[M + Na]^+$ calculated 844.50, found 844.48. ^{1}H NMR (500 MHz, CH_3OH-d_4): δ 6.35–6.18 (8H, m), 5.84 (2H, m), 5.70 (2H, m), 4.60 (1H, s), 4.30 (4H, s), 4.09 (2H, s), 3.66 (2H, s), 3.24 (9H, s), 2.72 (6H, s), 2.36–2.02 (4H, m), 1.73 (6H, s), 1.66 (6H, m), 1.51 (4H, m), 1.30–1.14 (4H, m), 1.05 (12H, s). ^{13}C NMR (75 MHz, CH_3OH-d_4): δ 148.41, 142.73, 138.39, 131.35, 131.06, 129.97, 128.76, 118.65, 116.85, 67.57, 60.98, 54.33, 49.01, 48.44, 41.03, 35.40, 33.99, 29.75, 22.12, 20.42, 14.37, 13.05.

The equilibrium solubility of Di-ATRA-PC conjugate was measured *via* an external standard method by HPLC analysis (as shown below). Firstly, standard samples with different concentration of Di-ATRA-PC conjugate were accurately prepared in water and stirred overnight at 37 °C. The standard curve and curve-fitted equation (data was omitted) were obtained by recording the integration of the peak areas of different concentrations of standard samples. Subsequently, an excessive solution was shaked for 3 h at 37 °C. After centrifugation, the equilibrium solubility was determined by measuring the amount of ATRA dissolved in water by HPLC according to the curve-fitted equation.

2.3. HPLC Analysis of Di-ATRA-PC Conjugate

Chromatographic detection was carried out by using Agilent 1100 high performance liquid chromatograph (Agilent, Palo Alto, CA) at 254 nm. Analyses were performed by using a Hypersil silica column (Analytical, 4.6 × 250 mm) at 25 °C with a mobile phase of methanol containing 0.1% TFA at a constant flow rate of 0.5 mL/min for 18 min. The sample volume for each injection was 20 μL. The retention time for ATRA and Di-ATRA-PC conjugate were 5.4 min and 6.3 min, respectively.

2.4. Critical Aggregation Concentration (CAC) of Di-ATRA-PC Conjugate

The self-assembly behavior of Di-ATRA-PC conjugate was assessed by electrical conductivity method (Zhu et al., 2015). Initially, Di-ATRA-PC conjugate at a concentration of 100 μg/mL was dissolved in water and diluted to different working concentrations ranging from 5 to 60 μg/mL. The conductivity of obtained solutions was detected by DDSJ-308F conductivity meter (INESA, Shanghai, China) at 25.0 ± 0.1 °C. The CAC value of Di-ATRA-PC solutions was examined at the break point of conductivity *versus* concentration curve.

2.5. Preparation of Di-ATRA-PC Liposomes

Di-ATRA-PC liposomes were prepared by thin film hydration method (Pedersen et al., 2010). In detail, 10 mg Di-ATRA-PC conjugate was dissolved in 5 mL of dichloromethane/methanol (v/v, 1/1) mixture in a round-bottom flask. The organic solvents were removed under evaporation and further dried with constant flow of nitrogen for 30 min to form dried thin lipid film. Subsequently, the lipid film was hydrated